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MODULATION OF APOPROTEIN E SECRETION IN RESPONSE TO RECEPTOR-MEDIATED ENDOCYTOSIS IN RESIDENT AND INFLAMMATORY MACROPHAGES

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The secretion by mononuclear phagocytes of apoprotein E (ApoE),[1] an M_r 33,000 protein that is a component of plasma lipoproteins, is regulated by multiple pathways. During development mononuclear phagocytes are derived from bone marrow precursors, circulate in the blood as monocytes, and arrive at tissues to become tissue macrophages; the appearance of ApoE secretion correlates with the acquisition of mature macrophage phenotype (2). Once present in tissues, macrophages may stay relatively quiescent as resident macrophages or become activated to kill microorganisms or tumor cells. Mature macrophages also regulate ApoE secretion. Although resident macrophages secrete abundant ApoE (up to 25% of total protein secretion), the secretion of ApoE is suppressed in activated macrophages (<2% of total protein secretion) (3, 4). Several of the regulatory stimuli, including cholesterol loading with acetylated low-density lipoprotein (AcLDL) (5, 6) and interaction with bacterial lipopolysaccharide endotoxin (4), involve a receptor-ligand interaction as an initial event; however, the relationship of these receptors to ApoE expression is not clear.

Macrophages endocytose particles and macromolecules by interaction with specific receptors for the Fc portion of immunoglobulin (7), complement fragments (8), acetylated and maleylated proteins (9), α_2-macroglobulin-proteinase complexes (10), and mannose- or fucose-terminated compounds (11), as well as nonspecific receptors (12). The binding of ligands to these receptors initiates a program of metabolic alteration, including production of reactive metabolites of oxygen and arachidonic acid (12, 13) and modulation of production of neutral proteinases such as plasminogen activator (14–16), cytolytic proteinase (16),

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[1] *Abbreviations used in this paper*: AcLDL, acetylated low-density lipoprotein; ApoE, apoprotein E; BCG, bacillus Calmette-Guerin; DME-LH, Dulbecco's modified Eagle's medium supplemented with lactalbumin hydrolysate; EIgG, sheep erythrocytes coated with IgG fraction of rabbit anti-erythrocyte antibody; EIgMC, sheep erythrocytes coated with IgM fraction of rabbit anti-erythrocyte antibody plus mouse complement; p18, p55, p62, p68, polypeptides of M_r 18,000, 55,000, 62,000, 68,000, respectively; SDS, sodium dodecyl sulfate.

elastase (15, 17), and collagenase (18). Because there is some correlation between the secretion of neutral proteinases and secretion of ApoE by macrophages (2–4), it was of interest to examine whether endocytic stimuli would modulate the secretion of ApoE in culture.

Materials and Methods

Macrophages were obtained from unstimulated mice or from mice injected with $NaIO_4$, pyran copolymer, or bacillus Calmette-Guerin (BCG) and cultured as described previously (3). For experiments on receptor-mediated modulation of macrophage secretion, macrophages were incubated with one of the following substances: AcLDL (19), IgG-coated sheep erythrocytes (EIgG) or complement-coated sheep erythrocytes (EIgMC) (both prepared as described previously [15]), dextran sulfate (Pharmacia Fine Chemicals, Piscataway, NJ), latex particles (Dow Chemical Co., Indianapolis, IN), or zymosan (yeast cell walls, ICN-K&K Laboratories Inc., Plainview, NY). Macrophages were radiolabeled with [^{35}S]methionine, and cellular and secreted proteins were analyzed after sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis as described previously (3). ApoE was identified by molecular size and by immunoprecipitation (3). The polypeptides p62 and p68 were identified by molecular size. Amounts of specific proteins were quantified by densitometry of fluorograms.

Results

ApoE Secretion Is Regulated Pleiotypically in Response to Endocytosis of AcLDL in Macrophages in Different States of Activation. AcLDL increased ApoE secretion by resident macrophages in a dose-dependent manner (Fig. 1, Table I). The maximum increase in ApoE secretion per cell of ≤fivefold was seen at 4.8 μg/ml AcLDL. AcLDL also increased the total incorporation of [^{35}S]methionine into secreted proteins; therefore, ApoE secretion expressed as a percentage of total secreted proteins remained constant (21–25%). In contrast to its effect on resident macrophages, AcLDL did not induce secretion of ApoE by BCG-activated macrophages, even though it increased total protein secretion by BCG-

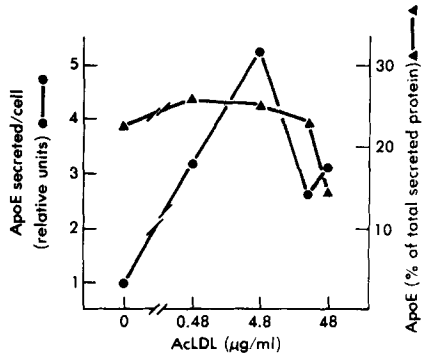

FIGURE 1. Dose response of stimulation of ApoE secretion in resident macrophages by AcLDL. Macrophages (1×10^6) were incubated with AcLDL in Dulbecco's modified Eagle's medium supplemented with lactalbumin hydrolysate (DME-LH) for 48 h and then labeled with [^{35}S]methionine for 2 h. Equal volumes of samples were applied to each lane, and the secreted proteins were separated by SDS-polyacrylamide gel electrophoresis. Secretion of ApoE was quantified by densitometry of fluorograms and is expressed as amount per cell in relative units (ratio to secretion by untreated macrophages) and as a percentage of total secreted proteins.

TABLE I
Secretion of ApoE after Endocytosis of AcLDL by Macrophages in Various States of Activation

Macrophage type	Ligand	ApoE secreted	
		Percent of total secreted proteins	Per cell (relative units)
Resident	None	22.5	1
	AcLDL	25.0	5.3
Periodate-elicited	None	6.3	1
	AcLDL	1.5	0.4
BCG-activated	None	1.1	1
	AcLDL	1.4	1.6

Resident, periodate-elicited, or BCG-activated macrophages (5×10^5) were incubated with AcLDL (24 μg/ml) for 48 h in DME-LH, then labeled with [^{35}S]methionine for 2 h. The secreted proteins were separated by SDS-polyacrylamide gel electrophoresis. Secretion of ApoE was quantified by densitometry of fluorograms and is expressed as a percentage of total secreted proteins and as amount per cell in relative units (ratio to secretion by untreated macrophages).

activated macrophages. AcLDL decreased the secretion of ApoE by periodate-elicited macrophages, which secrete amounts intermediate to those of resident macrophages and BCG-activated macrophages, and increased total protein secretion by these cells (Table I). That AcLDL was taken up to a similar extent by all three types of macrophages was shown by the accumulation of lipid droplets and by the fact that the secretion of other proteins was affected in similar ways in all macrophages (as shown subsequently). In addition to the increase of total incorporation of [^{35}S]methionine into secreted proteins within 4 h of incubation, AcLDL increased total incorporation of [^{35}S]methionine into cellular proteins. The increase in total synthesis of cellular proteins was twice that of the secreted proteins in both resident and periodate-elicited macrophages.

Dextran Sulfate Decreases ApoE Secretion. Basu et al. (5, 6) showed that the stimulation of ApoE secretion by AcLDL in resident and thioglycollate-elicited macrophages was due to the loading of cholesterol into macrophages rather than to the interaction of AcLDL with receptors. We found that dextran sulfate, which is recognized by the same receptor as AcLDL (9) but does not contain cholesterol, did not stimulate ApoE secretion by resident macrophages (Table II). Instead, dextran sulfate decreased the secretion of ApoE by all macrophage types.

Uptake of EIgG Affects ApoE Secretion Differently in Macrophages in Different States of Activation. When macrophages take up EIgG by Fc receptors, the cholesterol content within macrophages increases ≤fourfold (20). Therefore, we examined whether uptake of EIgG would affect ApoE secretion by macrophages. The effect of EIgG was dependent on the state of activation of macrophages (Table III). Secretion of ApoE by resident macrophages was slightly reduced by EIgG, and secretion by periodate-elicited macrophages was reduced to 5% of control. BCG-activated macrophages secreted little ApoE and did not respond significantly to EIgG.

TABLE II

Secretion of ApoE after Endocytosis of Dextran Sulfate by Macrophages in Various States of Activation

Macrophage type	Ligand	ApoE secreted	
		Percent of total secreted proteins	Per cell (relative units)
Resident	None	21.0	1
	Dextran sulfate	7.4	0.35
Periodate-elicited	None	23.1	1
	Dextran sulfate	6.8	0.29
BCG-activated	None	1.1	1
	Dextran sulfate	0.7	0.63

Resident, periodate-elicited, or BCG-activated macrophages (5×10^5) were incubated with dextran sulfate (10 μg/ml) for 48 h in DME-LH, then labeled with [^{35}S]methionine for 2 h. The secreted proteins were separated by SDS–polyacrylamide gel electrophoresis. Secretion of ApoE was quantified by densitometry of fluorograms and is expressed as a percentage of total secreted proteins and as amount per cell in relative units (ratio to secretion by untreated macrophages).

TABLE III

Secretion of ApoE after Endocytosis of EIgG by Macrophages in Various States of Activation

Macrophage type	Ligand	ApoE secreted	
		Percent of total secreted proteins	Per cell (relative units)
Resident	None	21.0	1
	EIgG	18.9	0.9
Periodate-elicited	None	11.5	1
	EIgG	0.6	0.05
BCG-activated	None	1.1	1
	EIgG	0.6	0.55

Resident, periodate-elicited, or BCG-activated macrophages (5×10^5) were incubated with EIgG (5×10^7) for 48 h in DME-LH, then labeled with [^{35}S]methionine for 2–4 h. The secreted proteins were separated by SDS-polyacrylamide gel electrophoresis. Secretion of ApoE was quantified by densitometry of fluorograms and is expressed as a percentage of total secreted proteins and as amount per cell in relative units (ratio to secretion by untreated macrophages).

ApoE Secretion Is Regulated by Uptake of Latex, Zymosan, and EIgMC. Ingestion of latex and zymosan decreased the secretion of ApoE by resident, periodate-elicited, and BCG-activated macrophages (Table IV). EIgMC, which are bound but not ingested by resident macrophages via the complement receptor (21), reduced the secretion of ApoE by resident macrophages to 60% of control (not shown).

Macrophages Show Receptor-Specific Alterations in Other Proteins. In addition to changes in ApoE, the endocytic stimuli produced various changes in other proteins. The pattern of cellular proteins did not change appreciably in response to most of the endocytic stimuli (Fig. 2). The exception was a band of M_r 18,000

TABLE IV

Secretion of ApoE after Endocytosis of Latex and Zymosan by Macrophages in Various States of Activation

Macrophage type	Ligand	ApoE secreted	
		Percent of total secreted proteins	Per cell (relative units)
Resident	None	21.0	1
	Latex	1.6	0.08
	Zymosan	0	0
Periodate-elicited	None	23.1	1
	Latex	2.5	0.11
	Zymosan	0.6	0.03
BCG-activated	None	1.1	1
	Latex	0.6	0.55
	Zymosan	0.2	0.18

Resident, periodate-elicited, or BCG-activated macrophages (5×10^5) were incubated with latex (50 μg) or zymosan (25 μg) for 48 h in DME-LH, then labeled with [^{35}S]methionine for 2 h. The secreted proteins were separated by SDS-polyacrylamide gel electrophoresis. Secretion of ApoE was quantified by densitometry of fluorograms and is expressed as a percentage of total secreted proteins and as amount per cell in relative units (ratio to secretion by untreated macrophages).

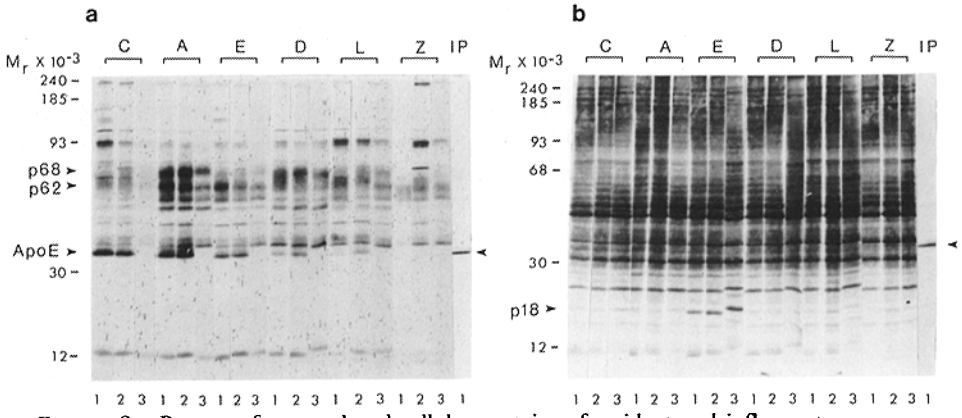

FIGURE 2. Pattern of secreted and cellular proteins of resident and inflammatory macrophages incubated with various endocytic stimuli. 5×10^5 resident (1), periodate-elicited (2), or BCG-activated (3) macrophages were incubated in 1 ml of control medium alone (*C*), with 24 μg AcLDL (*A*), 5×10^7 EIgG (*E*), 10 μg dextran sulfate (*D*), 50 μg latex (*L*) or 25 μg zymosan (*Z*) for 48 h in DME-LH. After washing, macrophages were labeled with [^{35}S]methionine for 2 h, and the secreted (*a*) and cellular (*b*) proteins were analyzed by SDS-polyacrylamide gel electrophoresis. Approximately the same amount of radioactivity was applied to each lane of gel. Secreted and cellular ApoE was also immunoprecipitated from control macrophages (*IP*). M_r ($\times 10^{-3}$) markers and migration of polypeptides p68, p62, p18, and ApoE are indicated.

(p18), which became prominent only in EIgG-treated cells. In contrast, the pattern of secreted proteins changed markedly, and the changes were specific, depending on the endocytic stimuli and on the state of activation of the macrophages. The polypeptides of apparent M_r 62,000 and 68,000 (p62 and p68), which account for 14–20% and 0–6%, respectively, of the secretory products of untreated macrophages, were increased most dramatically by many endocytic

stimuli. The p62 secretion was increased ≤2–3-fold in resident and periodate-elicited macrophages by AcLDL, EIgG, latex, and zymosan but not by dextran sulfate. BCG-activated macrophages did not alter p62 secretion in response to any stimuli.

The secretion of p68 in all three types of macrophages was increased by both AcLDL and dextran sulfate, which are recognized by similar receptors (9), approaching 25% of the total secreted proteins, whereas EIgG did not increase p68. The only other ligand that increased p68 was latex, in BCG-activated but not in other macrophages. The untreated macrophages had a distinct protein band between p62 and p68 that accounted for 10–14% of the secretory products, but when p62 or p68 increased, this band seemed to disappear. Both p62 and p68 were broad bands, which suggested that they were heavily glycosylated or that they comprised several unresolved polypeptides. Because p62 and p68 had electrophoretic mobilities that varied slightly, it is possible that several different receptor-specific proteins were induced. For example, zymosan-induced p62 seemed to have a slightly lower molecular weight than AcLDL-, EIgG-, and dextran sulfate-induced p62, and latex-induced p62 seemed to have a slightly higher molecular weight than the p62 induced by any of the other endocytic stimuli.

In addition to these changes, the endocytic stimuli showed changes in other bands specific for particular stimuli. AcLDL increased the two bands electrophoresed below p62, approximately M_r 55,000 and 51,000 (p55 and p51), and decreased a doublet at M_r 93,000 (p93) that is probably complement factor B (22). Dextran sulfate also increased p51 but not p55. Latex and zymosan kept p93 at a high secretion level in all macrophage types, but most other stimuli decreased it. Concomitant with the decrease in ApoE secretion caused by dextran sulfate, latex, and zymosan, a band of M_r 37,000 became prominent, although this protein was not immunoprecipitable by anti-ApoE antibody. This band was also prominent in BCG-activated macrophages regardless of treatment.

Effects of EIgG on Cellular and Secreted Proteins of Macrophages Are Regulated by

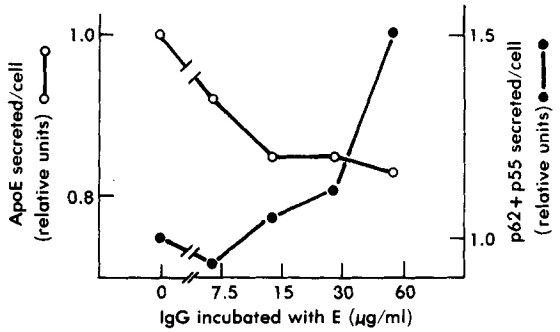

FIGURE 3. Modulation of p62, p55, and ApoE secretion in resident macrophages by sheep erythrocytes coated with IgG. Erythrocytes (*E*) were incubated with anti-erythrocyte antibody of various concentrations. EIgG (5×10^7) thus prepared were incubated with macrophages (1×10^6) for 16 h in DME-LH, then labeled with [^{35}S]methionine for 4 h. The secreted proteins were separated by SDS-polyacrylamide gel electrophoresis. Secretion of ApoE and the combined secretion of p62 and p55 were quantified by densitometry of fluorograms and are expressed as amount per cell in relative units (ratio to secretion by untreated macrophages).

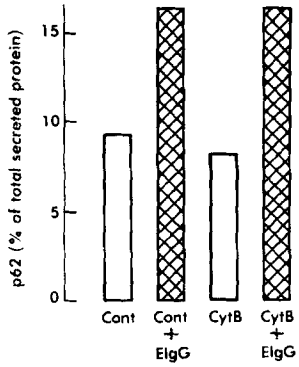

FIGURE 4. Effect of cytochalasin B on secretion of p62 induced in resident macrophages by EIgG. Macrophages (1×10^6) were incubated with EIgG (5×10^7) for 24 h in DME-LH in the presence of 2 μg/ml cytochalasin B dissolved in dimethyl sulfoxide at a final concentration of 0.16%, then labeled with [^{35}S]methionine for 4 h in the presence of cytochalasin B. The secreted proteins were separated by SDS–polyacrylamide gel electrophoresis. Secretion of p62 was quantified by densitometry of fluorograms and is expressed as a percentage of total secreted proteins. *Cont*, macrophages incubated without cytochalasin B; *Cont + EIgG*, macrophages incubated with EIgG but without cytochalasin B; *CytB*, macrophages incubated with cytochalasin B; *CytB + EIgG*, macrophages incubated with EIgG and cytochalasin B.

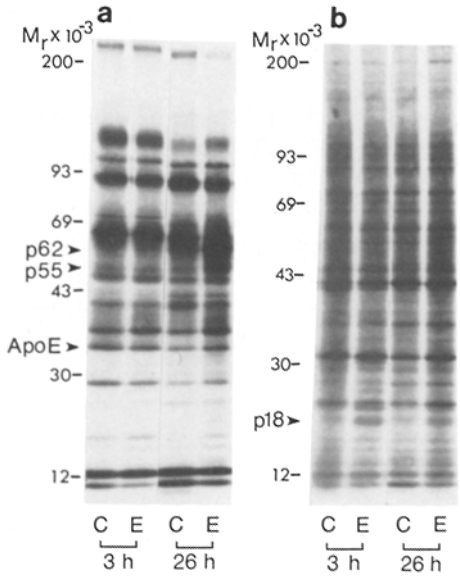

FIGURE 5. Secreted and cellular proteins of resident macrophages incubated with EIgG. Macrophages (1×10^6) were incubated with EIgG (1×10^8) for 3 or 26 h in DME-LH, then labeled with [^{35}S]methionine for 4 h. The secreted (*a*) and cellular (*b*) proteins were analyzed by SDS-polyacrylamide gel electrophoresis. *C*, control macrophages; *E*, macrophages incubated with EIgG. M_r ($\times 10^{-3}$) markers and migration of polypeptides p62, p55, and ApoE were indicated.

Fc Receptors. The increase of p62 and p55 and the decrease of ApoE observed in resident macrophages were dependent on both the number of EIgG given to each macrophage and the amount of IgG coating each erythrocyte (Fig. 3). When macrophages were incubated with EIgG in the presence of cytochalasin

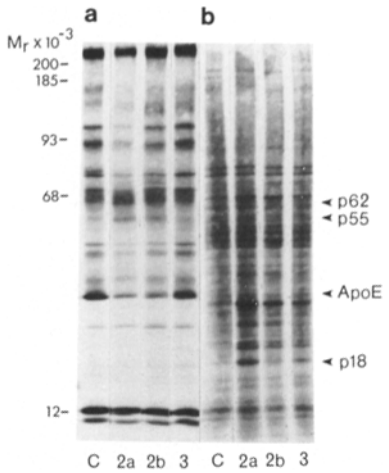

FIGURE 6. Effect of IgG isotypes on secreted and cellular proteins of resident macrophages. Macrophages (1×10^6) were incubated with erythrocytes (1×10^8) coated with monoclonal anti-erythrocyte antibody of IgG_{2a}, IgG_{2b}, or IgG_3 subclasses ($EIgG_{2a}$, $EIgG_{2b}$, $EIgG_3$), then labeled with [^{35}S]methionine for 4 h. The secreted (*a*) and cellular (*b*) proteins were analyzed by SDS-polyacrylamide gel electrophoresis. At 3 h of incubation, the percentage of macrophages with EIgG rosettes for each subclass was IgG_{2a}, 90%; IgG_{2b}, 100%; and IgG_3, 15%. M_r ($\times 10^{-3}$) markers and migration of polypeptides p62, p55, p18, and ApoE are indicated. *C*, macrophages incubated without EIgG.

B, which inhibits the ingestion phase but not the binding phase of phagocytosis (23), the increase in p62 was not inhibited by cytochalasin B. This indicates that ingestion was not necessary for the induction of changes in the protein secretion pattern by EIgG (Fig. 4). To induce changes in secreted proteins, continuous incubation of EIgG and macrophages for 24 h was necessary; incubation of ligands and macrophages for 3 h was not effective (Fig. 5). However, the induction of p18 in cellular proteins was seen after as little as 3 h of incubation.

Because mouse macrophages have three subclass-specific Fc receptors (IgG_{2a}, IgG_{2b}/IgG_1, and IgG_3) (7), we determined which subclass-specific receptors stimulate altered secretion by using erythrocytes coated with IgG_{2a}, IgG_{2b}, and IgG_3 subclasses of monoclonal anti-erythrocyte antibody. Both IgG_{2a} and IgG_{2b} caused alterations typical of EIgG; they decreased ApoE secretion, increased p62 and p55 secretion, and induced cellular p18 (Fig. 6). Although IgG_3 showed only slight changes in any of these bands, this ligand did not bind as well as the other two subclasses under our conditions, and we could not draw definite conclusions about its effects.

Discussion

We have shown here that receptor-mediated binding and uptake of a variety of substances modulate secretion of ApoE and that the response depends both on the nature of the ligand and on the functional state of the target macrophages. These endocytic stimuli altered other secretory or cell-associated proteins as well, including p62, p68, and p18, which are particularly susceptible to modulation by endocytosis. Although there were common responses to a variety of

endocytic stimuli, each ligand had its own specific effects.

We have also demonstrated that the response of macrophages to AcLDL depends on their state of activation. Previously, Basu et al. (5, 6) showed that resident and thioglycollate-elicited macrophages increased ApoE and total protein secretion in response to AcLDL and that this was due to the increased cholesterol content in macrophages. We confirmed that AcLDL increases ApoE and total protein secretion of resident macrophages in a dose-dependent manner and found that dextran sulfate, which is recognized by the same receptor as AcLDL but does not contain cholesterol, did not increase ApoE and total protein secretion. However, we found that BCG-activated macrophages, which secrete little ApoE, did not respond to AcLDL and that periodate-elicited macrophages, which secrete an intermediate level of ApoE, responded to AcLDL by decreasing ApoE secretion. Both BCG-activated and periodate-elicited macrophages took up AcLDL to a similar extent, as judged by the accumulation of lipid droplets in the cells, and AcLDL increased the total incorporation of [^{35}S]methionine into the secreted proteins to a similar extent in both macrophage types. That the BCG-activated macrophages were not generally unresponsive to endocytosis was shown by the fact that they changed their pattern of protein synthesis in response to various endocytic stimuli. Therefore, our observations suggest that whether macrophages secrete ApoE after ingestion of cholesterol is regulated by their functional state.

EIgG, which are recognized by three distinct isotype-specific Fc receptors of macrophages, increase the cellular content of cholesterol as AcLDL does. However, EIgG did not increase ApoE secretion by resident macrophages, but rather reduced it slightly. EIgG did not alter the low secretion of BCG-activated macrophages and markedly reduced ApoE secretion in periodate-elicited macrophages. In all macrophages EIgG increased the synthesis of cellular p18 and increased secretion of p62 and p55. The secretion of p62 was related to the triggering of a receptor at the plasma membrane level rather than by ingestion, because cytochalasin B, which prevents ingestion but not binding of EIgG, did not inhibit the induction of p62. Many endocytic ligands, including dextran sulfate, EIgG, EIgMC, latex, and zymosan, reduced the secretion of ApoE by macrophages. Because decreased secretion of ApoE is a phenotype associated with activation of macrophages, our findings suggest that many endocytic stimuli activate macrophages in vitro. We and others previously showed that endocytosis increases secretion of elastase and plasminogen activator (15, 17). The secretion of elastase is characteristic of stimulated macrophages but not of activated macrophages. Therefore, endocytosis may bring macrophages to a special state of activation.

Several possible mechanisms that regulate the secretion of macrophages by endocytosis have been proposed, including (*a*) stimulation by triggering of the respiratory burst (24), (*b*) stimulation caused by storage of undigestible material in secondary lysosomes (17, 18), and (*c*) release of ingested material into the cytoplasm (5, 6). Although the increased secretion of ApoE induced by AcLDL in resident and thioglycollate-elicited macrophages has been shown to be due to the accumulation of cholesterol in the cells, i.e., the third suggested mechanism, the accumulation of cholesterol caused by EIgG did not induce increased secre-

tion of ApoE in resident macrophages. Therefore, the route of entry of cholesterol may also modulate this effect. In addition, AcLDL decreased ApoE secretion by periodate-elicited macrophages, and many other endocytic stimuli decreased ApoE secretion by macrophages in various states of activation. This reduction of ApoE secretion may be related to the storage of material in secondary lysosomes, because dextran sulfate, latex, and zymosan are all undigestible by macrophages, and EIgG also stays transiently in secondary lysosomes. The reduction of ApoE secretion is not likely to be mediated by the triggering of the respiratory burst, as has been proposed for the secretion of plasminogen activator (24), because 12-O-tetradecanoylphorbol-13-acetate, which triggers the respiratory burst, does not affect ApoE secretion (2). Thus, as part of their pleiotypic response, receptors mediating the scavenger function of macrophages also serve to regulate secretion. The exact nature of the signals involved in the regulation of macrophage secretion by endocytosis and the diversity of the macrophage response remains to be determined.

Summary

We have determined the effect of various endocytic ligands on the secretion of ApoE by macrophages. ApoE was a major secreted protein of resident macrophages, but BCG-activated macrophages secreted little ApoE and periodate-elicited macrophages secreted intermediate amounts of ApoE. Resident, periodate-elicited, and BCG-activated mouse peritoneal macrophages were incubated with AcLDL, EIgG, EIgMC, dextran sulfate, latex, or zymosan, and the resulting protein secretion patterns were analyzed by [^{35}S]methionine labeling and SDS-polyacrylamide gel electrophoresis. AcLDL increased total [^{35}S]methionine incorporation into secreted proteins. Although AcLDL increased the secretion of ApoE by resident macrophages ≤fivefold in a dose-dependent manner, with maximal stimulation at 4.8 μg/ml, it decreased the secretion of ApoE by periodate-elicited macrophages to almost nothing and did not affect the low rate of secretion of ApoE by BCG-activated macrophages. However, EIgG, which increases cellular cholesterol content of macrophages as AcLDL does, did not increase ApoE secretion, and dextran sulfate, which is recognized by the same receptor as AcLDL, also did not increase ApoE secretion. The binding and uptake of EIgG, dextran sulfate, zymosan, latex, and EIgMC all decreased the secretion of ApoE.

These endocytic ligands also altered the pattern of secreted and cellular proteins other than ApoE. The pattern of response was ligand-specific. However, increased secretion of polypeptides of M_r 62,000 and 68,000 was common to many stimuli. We conclude that receptor-mediated endocytosis modulates the secretion of ApoE and other proteins pleiotypically in resident, inflammatory, and activated macrophages.

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